Plant Physiol. (1991) 95, 509-516
0032-0889/91/95/0509/08/$01.00/0

Received for publication August 2, 1990
Accepted October 29, 1990

The Role of Glutamate Dehydrogenase in Plant Nitrogen Metabolism[1,2]

Sharon A. Robinson*, Annette P. Slade, Gary G. Fox, Richard Phillips, R. George Ratcliffe, and George R. Stewart

Biology Department, University College London, Gower Street, London WC1E6BT (S.A.R., A.P.S., R.P., G.R.S.); and Department of Plant Sciences, Oxford University, Agricultural Science Building, Parks Road, Oxford OX1 3PF (G.G.F., R.G.R.)

ABSTRACT

***In vivo* nuclear magnetic resonance spectroscopy, *in vitro* gas chromatography-mass spectrometry, and automated $^{15}N/^{13}C$ mass spectrometry have been used to demonstrate that glutamate dehydrogenase is active in the oxidation of glutamate, but not in the reductive amination of 2-oxoglutarate. In cell suspension cultures of carrot (*Daucus carota* L. cv Chantenay), primary assimilation of ammonium occurs via the glutamate synthase pathway. Glutamate dehydrogenase is derepressed in carbon-limited cells and in such cells the function of glutamate dehydrogenase appears to be the oxidation of glutamate, thus ensuring sufficient carbon skeletons for effective functioning of the tricarboxylic acid cycle. This catabolic role for glutamate dehydrogenase implies an important regulatory function in carbon and nitrogen metabolism.**

The GOGAT[3] cycle is now widely accepted as the major route of ammonium assimilation in higher plants. Studies of primary ammonium assimilation, the photorespiratory nitrogen cycle, and secondary ammonium assimilation (that derived from the catabolism of nitrogenous storage and transport compounds) have all yielded results consistent with the consecutive action of GS and GOGAT (6, 20, 28). However, controversy still exists as to the role of the enzyme GDH in higher plants. It is found in all higher plants examined and is often present at high levels in, for example, senescing and root tissues (11, 22). The enzyme could operate primarily in the assimilation or reassimilation of ammonium and play a complementary role to the GOGAT cycle (18, 23, 29). Alternatively, it could catalyze the oxidation of glutamate and furnish carbon to the TCA cycle (26). The general characteristics of the enzyme offer conflicting evidence with regard to its role. *In vitro* the thermodynamically favored direction of the reaction is the production of glutamate, but the enzyme is reported as having a very large Michaelis constant with respect to ammonium (24), a characteristic that argues strongly against an assimilatory role (14). However, the large amounts of GDH present in some tissues may be adequate to account for observed rates of ammonium assimilation, even if intracellular substrate concentrations of ammonium are low. It is suggested that GDH has a role in ammonium reassimilation under conditions of stress (23). However, attempts to confirm an assimilatory role, using inhibitors of GS and GOGAT have failed to show ammonium assimilation under conditions where the GOGAT cycle is blocked. Inhibition of GS by MSO brings about an accumulation of ammonium and stops the incorporation of ^{15}N-labeled ammonium into amino acids (2, 6, 20). There are reports of relatively small amounts of incorporation via an MSO insensitive pathway (18), but these represent less than 1% of total ammonium incorporated. Assuming that MSO does not affect the activity of GDH, and this assumption has been questioned (18), the general conclusion from these experiments is that GDH plays no role in ammonium assimilation. The present investigation was undertaken to establish if the function of GDH is the catabolism of glutamate.

MATERIALS AND METHODS

Growth Conditions

Suspension cultures of carrot, *Daucus carota* L. cv Chantenay, isolated 6 years previously were maintained on Murashige and Skoog (15) medium (Flow Labs, Irvine, Scotland), supplemented with 2,4-D (0.2 mg/L), kinetin (0.1 mg/L), and sucrose (2% w/v) (MDK), in 250 mL Erlenmeyer flasks at 25°C. Cells were subcultured at 14 d intervals by inoculating 7 mL of suspension into 70 mL of fresh medium.

Enzyme Extraction and Assays

Cells were collected by filtration on Whatman No. 1 paper, washed with 25 mL distilled water and weighed. Cells (0.2 g) were ground in liquid N_2 and extracted in 5 mL buffer. The

[1] S. A. R. and A. P. S. were supported the Science and Engineering Research Council. G. G. F. was supported by a Science and Engineering Council CASE studentship with Unilever Research. R. G. R. acknowledges the financial support of the Agricultural and Food Research Council.

[2] This paper is dedicated to the memory of Dr. A. P. Sims who initiated the use of ^{15}N isotopes in studies of plant nitrogen metabolism.

[3] Abbreviations: GOGAT, glutamate synthase; GS, glutamine synthetase; GABA, γ-aminobutyric acid; GDH, glutamate dehydrogenase; MSO, methionine sulfoximine; AOA, aminooxyacetic acid; ANCA, automated $^{15}N/^{13}C$ analyzer; MTBSTFA, *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide; HFIB, heptafluorbutyryl isobutyl; t.BDMS, *tert*-butyldimethylsilyl; INEPT, insensitive nuclei enhanced by polarization transfer.

GS/GDH extraction buffer (pH 8.0) contained 25 mM Tris, 1 mM EDTA, 1 mM DTT, 1 mM mercaptoethanol, 1 mM reduced glutathione, 10 mM $MgSO_4$, 5 mM glutamate, 2% PVP, and 0.01% Triton. The GOGAT extraction buffer (pH 7.2) contained 100 mM sodium phosphate, 10% v/v glycerol, 5 mM EDTA, 0.1% Triton-X-100, 5 mM mercaptoethanol, and 1 mM PMSF. After centrifugation, to remove cell debris, the supernatants were used for the enzyme and soluble protein assays described below.

GS (EC 6.3.1.2) was determined by the synthetase assay. The reaction mixture contained: 15 μmol ATP, 20 μmol $MgSO_4$, 5 μmol hydroxylamine, 60 μmol L-glutamate, and 37.75 μmol Tris (final pH 7.6 and final volume 0.75 mL). The reaction was initiated by the addition of 0.25 mL enzyme extract and terminated after 30 min by the addition of 0.75 mL ferric chloride reagent. After centrifugation the absorbance at 500 nm was determined.

GOGAT (EC 2.6.1.53) was assayed by determining the rate of glutamine-dependent NADH oxidation. The reaction mixture contained 5 μmol L-glutamine, 5 μmol 2-oxoglutarate, 0.08 μmol NADH, 5 μmol EDTA, and 50 μmol Tricine buffer (final pH 7.5 and final volume 1 mL).

GDH (EC 1.4.1.2) was assayed by determining the rate of 2-oxoglutarate-dependent NADH oxidation. The reaction mixture contained 150 μmol NH_4Cl, 1 μmol $CaCl_2$, 0.3 μmol NADH, 20 μmol 2-oxoglutarate, and 100 μmol Tris buffer (final pH 8.2 and final volume 1 mL).

All enzyme assays were carried out at 30°C and were linear with respect to length of incubation time and quantity of enzyme assayed. Soluble protein was determined using the Bio-Rad protein assay (3).

Expression of Results

Enzyme activities are given as nanokatals per mg protein.

Protein, Ammonium, and Sucrose Determination

Total cell protein was extracted by alkaline hydrolysis (4). The sample was neutralized and assayed using the Bio-Rad protein assay.

Medium ammonium concentration was assayed directly using a colorimetric method (13). For determination of [^{15}N] ammonium in the medium the ammonium was first purified by cation exchange to remove all amino acids and to concentrate the sample. The medium was applied to a Bio-Rad 50X-Na+ membrane (Bio-Rad Laboratories, Hemel Hempstead, Herts), and after washing with 15 mL sodium acetate (10 mM) to remove amino acids, the ammonium was eluted with 1 mL 0.5 M sodium phosphate.

Medium sucrose concentration was determined using the phenol-sulfuric method (5).

^{15}N-Labeling Studies

^{15}N-labeling studies were performed on stationary phase cells because these exhibited the highest levels of GDH activity. After 12 d growth on MDK medium, cells were collected on a 75 μm mesh sieve, washed, resuspended in nitrogen-free media, and returned to the shaker. After 1 h the cells were centrifuged and resuspended in flasks containing the labeled nitrogen source and inhibitors.

For the ammonium treatment cells were grown for 24 h on media containing 2 mM $^{15}NH_4Cl$ (MSD Isotopes, Cambrian Gases, Croydon) in the presence and absence of 1 mM MSO. Samples were taken, at hourly intervals to 6 h and at 24 h, and centrifuged to separate the medium and the cells. The medium was analyzed for ammonium as described above. Cells were collected as for enzyme extraction (above) and were then extracted in methanol (0.5 g/10 mL) for amino acid analysis by GC-MS.

For the glutamate treatment, cells were grown for 24 h on 5 mM [^{15}N]glutamate in the presence and absence of 1 mM MSO or 1 mM AOA. Samples were taken at 0, 12, 18, and 24 h and analyzed as above. In addition total ^{15}N incorporation into cells was determined using the ANCA system. The media glutamate concentrations were determined by HPLC.

All experiments were repeated four times with good replication.

HPLC Determination of Soluble Amino Acid Pools

Cell amino acids were determined directly from the methanol extracts using HPLC. The amino acids were analyzed as *o*-phthaldialdehyde derivatives on a C-18 column using a method adapted from Joseph and Marsden (10). All samples, standards, eluents, and derivatizing reagents were passed through a 0.5 μm FP Vericel filter (Gelman Sciences Ltd., Northampton) prior to use. Ten microliters of 10 μL 0.25 mM homoserine (internal standard) and a 10 μL sample were derivatized with 60 μL working reagent. After 2 min 8 μL derivatized sample were injected. The gradient was produced using two eluents: A, 0.1 M phosphate buffer (pH 8) with 20 mL/L methanol and 20 mL/L tetrahydrofuran; B, 65% methanol. Eluents were degassed with helium prior to use. Gradient was programmed as follows; 0 to 5 min, 20% to 35% B; 5 to 27 min, 35% to 100% B; 27 to 32 min, 100% B.

The HPLC was fitted with a 2 cm guard column and a 10 × 0.45 cm column containing Spherisorb 5 μm ODS2 spherical packing (Phase-Sep Ltd, Deeside, UK).

ANCA Analysis of ^{15}N-Labeled Ammonium and Total Cell Enrichment

Total insoluble N was determined by the technique of ANCA-mass spectrometry using a Europa Scientific Roboprep-Tracermass system. After extraction of soluble N in methanol, cells were washed in distilled water and samples prepared and analyzed as described by Barrie and Lemley (1). Samples (5 mg) were analyzed in triplicate. Medium ammonium was analyzed after concentration (see above). Samples (40 μL) were absorbed onto Carbosorb and analyzed in triplicate using the small sample mode (1). All samples were calibrated against standards containing 15 atom % $^{15}NH_4Cl$.

GC-MS Analysis of [^{15}N]Amino Acid Derivatives

Cell methanol extracts were taken to dryness by rotary evaporation, redissolved in 2 mL water and applied to a Bio-Rex sample preparation disc with AG 50W-X8 cation ex-

change resin, washed with 5 mL water and amino acids and amides were eluted with 5 mL 6 M NH_4OH. The amino acid fraction was lyophilized and redissolved in 1 mL 50% methanol. Purified extract (0.5 mL) was taken directly into a silanized glass vial, dried under nitrogen, and derivatized with MTBSTFA (Pierce Chemical Co.) as described by Fortier *et al.* (7). Derivatizing mixture (50 μL), MTBSTFA:pyridine:triethylamine (15:15:1 by volume), was added to the dry samples and the vials were heated at 75°C for 30 min. ^{15}N incorporation into each amino acid (including glutamine and asparagine) was then carried out by GC-MS analysis of the t.BDMS derivatives using a VG7070 H mass spectrometer linked to a Finnigan Incos data system. A sample of 0.2 to 0.4 μL was run on a Pye-Unicam 204 gas chromatograph with an all glass dropping-needle solid injector and fitted with a 25 M polydimethylsiloxane 0.25 μm film thickness fused silica capillary column. Helium was used as carrier gas at a column head pressure of 70 kPa, and the oven was temperature programmed from 120°C for 1 min, +4°C / min to 280°C. Mass spectra were acquired using an electron energy 70 eV, and the mass range scanned from m/z 750 to 35 every 2 s with a total cycle time of 3 s. Spectra obtained from t.BDMS derivatives generally show an intense ion at $(M-57)^+$ or $(M-159)^+$. ^{15}N incorporation (atom % excess) was calculated after integrating the areas obtained for $(M-57)^+$ for both labeled and unlabeled amino acids. t.BDMS derivatives allow the determination of the total amount of ^{15}N incorporated into glutamine and asparagine and the proportion of the amides that are singly or doubly labeled.

To determine the ^{15}N label in both the amide-N and the amino-N a second derivative was prepared. The remaining 0.5 mL amino acid extract was separated into neutral/basic and acidic amino acids by Dowex 1-acetate ion exchange chromatography (19). The neutral and basic amino acid fraction was lyophilized, redissolved in 50% methanol, dried under nitrogen, and derivatized as the HFIB esters as described by Rhodes *et al.* (19). On preparation of these derivatives the amide groups of glutamine and asparagine are lost to form glutamate and aspartate, respectively. GC-MS then allows direct determination of the ^{15}N incorporation into the amino-N of glutamine (determined as glutamate). GC/MS of the HFIB derivatives was performed as for t.BDMS derivatives but the mass range scanned was m/z 200 to 400 and the GC was temperature programmed from 120°C for 2 min, +6°C/ min to 280°C. The ^{15}N in amino-N of glutamine was calculated after integration of ions m/z 298,299. The ^{15}N label in amide-N of glutamine was then calculated by difference.

Preparation of Cells for *in Vivo* NMR

Carrot cells were cultured as described previously (8). Prestationary phase cells (11 d) were harvested by gentle filtration through a 40 μm nylon mesh. The cells (approximately 12 g fresh wt) were resuspended in nitrogen-free medium supplemented with 5 g/L sucrose at pH 5.6 to give a total volume of 25 mL, corresponding to a packed cell volume of 50%. The suspension was transferred to a 20 mm diameter NMR tube and cells were oxygenated with an airlift system operating with an oxygen flow rate of up to 50 mL/min (9). For experiments performed in a 10 mm diameter NMR tube, approximately 2.5 g fresh wt of cells were resuspended in 6 mL of medium and the suspensions oxygenated using a scaled down version of the airlift system operating at 20 mL/min. The cells were allowed to stabilize in the NMR tube for 3 h before the acquisition of spectra. Enzyme inhibitors were added to the suspending medium in the NMR tube immediately after the transfer of the cells and ^{15}N-labeled glutamate or ammonium chloride were added at the end of the 3 h equilibration period. The initial extracellular concentration was 2 mM for the inhibitors and 20 mM for the labeled glutamate and ammonium chloride.

^{15}N NMR Spectroscopy

^{15}N NMR spectra were recorded at 30.42 MHz on a Bruker CXP 300 spectrometer using a 10 or 20 mm diameter broad band frequency probe head. Cell suspensions were oxygenated continuously with the airlift system and the temperature was maintained at 25°C. ^1H-coupled ^{15}N NMR spectra were usually accumulated with a 90° pulse angle, a recycle time of 5 s, and a total acquisition time of 1 h for qualitative experiments and a 30° pulse angle, a 20 s recycle time, and a 4 h acquisition time for quantitative experiments. ^1H-decoupled NMR spectra were usually accumulated with a 90° pulse angle, a recycle time of 2 s, a total acquisition time of 1 h, and low power decoupling for 1.75 s prior to acquisition to produce the nuclear Overhauser enhancement and high power decoupling for 0.25 s during the acquisition. INEPT spectra were usually accumulated with a 90° pulse angle, a 2.35 s recycle time, and an acquisition time of 30 min. Chemical shifts are quoted relative to the resonance at 0 ppm from nitrate.

RESULTS AND DISCUSSION

Ammonium Assimilation

The activities of GS and GOGAT were highest during the period of rapid cell growth and maximum ammonium assim-

Table I. *Activities of GS, GOGAT, and GDH in Relation to Media Nitrogen and Sucrose Concentrations*

Data represent values for 2 d within the culture period. Days are numbered from the subculturing of cells (d 0). Values are the mean of three replicates ± SD.

Treatment	Enzyme Activity		
	GS	GOGAT	GDH
	nkat/mg protein		
Cells in exponential phase (d 5), high total nitrogen (50 mM), high sucrose (10 g/L)	3.0 ± 0.11	0.47 ± 0.01	6.67 ± 0.11
Cells in stationary, phase (d 14), high total nitrogen (45 mM), low sucrose (0.5 g/L)	0.67 ± 0.01	0.27 ± 0.006	23.34 ± 0.4

ilation which occurred during the first 10 days of the culture period (Table I). During this time the cells exhausted various nutrients, particularly sucrose, and entered a stationary phase where growth ceased. GS and GOGAT levels fell as the cells entered this phase and remained low until the cells were placed in fresh medium. Although sucrose was limiting during stationary phase the level of total nitrogen remained high. In contrast to GS, GDH levels were highest during the stationary phase and fell when cells were placed in fresh medium. During the period of rapid ammonium assimilation GDH levels remained low, but at the end of the culture period, when sucrose became limited, GDH levels increased by a factor of 3.5. GDH levels were high relative to GS throughout the culture period, but this differential was smallest (2:1) during the period of nitrogen metabolism and highest during sucrose limitation (35:1). Levels of GDH were up to ten times those measured in many plant tissues (17, 25). These results suggest that, while ammonium assimilation was occurring primarily via the GOGAT cycle, the high levels of GDH activity could also make a substantial contribution to ammonium assimilation. The relative flux through these two routes of assimilation were investigated by determining the incorporation of [^{15}N]ammonium in the presence and absence of MSO.

[^{15}N]ammonium incorporation was analyzed using *in vivo* NMR and GC-MS. Figure 1 shows the *in vivo* ^{15}N NMR spectra obtained in the presence of 20 mM [^{15}N]ammonium over a 10 h period. The label from the ammonium appeared in the amide-N of glutamine during the first hour and this pool increased steadily over a 3 h period before declining rapidly. INEPT experiments (data not shown) showed that the label appeared in the amide of glutamine within the first 30 min. Label was observed in the amino-N of glutamate during the second hour, increasing to a steady state by 4 h and remaining constant for the next 6 h of the experiment. Labeled alanine appeared after 3 h and increased over the remaining 7 h. GABA was labeled at 7 h and remained constant until 10 h. The sequence of spectral changes in Figure 1 is similar to that observed previously by *in vivo* ^{15}N NMR in the ectomycorrhizal fungus *Cenococcum graniforme* (12) and shoot-forming cultures of white spruce buds (27). Labeling of the amino acids did not occur in the presence of MSO. Thus, by using NMR it was possible to observe the incorporation of [^{15}N]ammonium into living cells, but this technique did not allow accurate determination of the metabolites with a good time resolution and detection was limited to the more abundant amino acids.

GC-MS was used to quantitatively determine the incorporation of label into amino acids. Incorporation of [^{15}N]ammonium was followed over a 24 h period in the presence and absence of MSO. In the control cultures GS activity remained constant over the 24 h period. Treatment with MSO produced a steady decline in GS activity and complete inhibition was observed after 5 h. GDH activity declined by 40% over the 24 h in both the control and the MSO treatment. This is consistent with the decline in GDH activity when cells were placed in fresh media.

In the control cells ^{15}N label was detected in most amino acids after 1 h and the incorporation of label (nmol/g fresh wt) increased up to 5 h. Table II therefore shows the maximum values obtained over the 24 h period. Results are given as

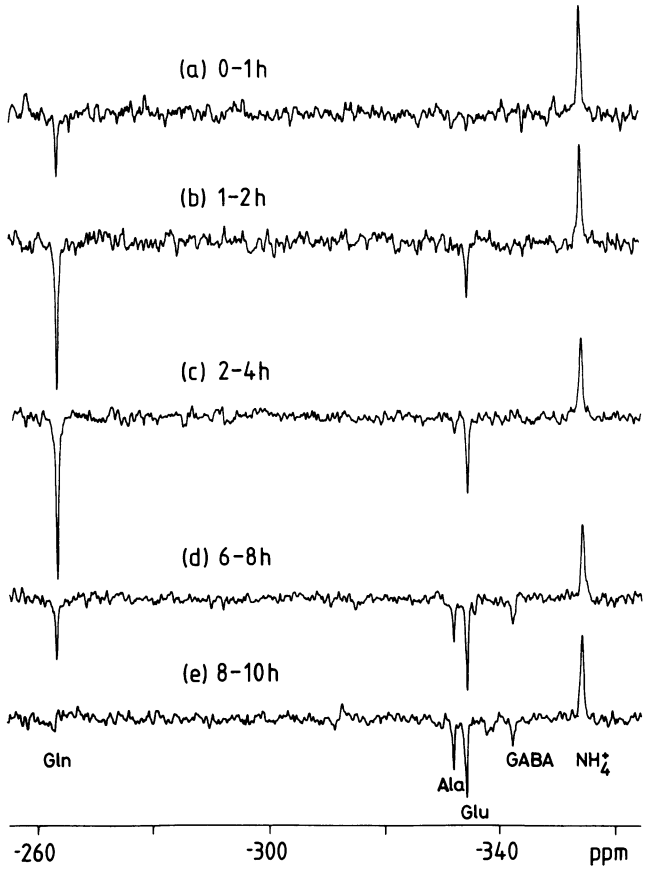

Figure 1. ^{1}H-decoupled ^{15}N NMR spectra of 11 d carrot cells following the addition of 20 mM [^{15}N]ammonium chloride. The spectra were obtained in 1 or 2 h over the time intervals indicated. The resonances were assigned on the basis of their chemical shift (12, 27). Several amino acids give an amino resonance at approximately −335 ppm, and it was necessary to use the GC-MS data to assign the resonance at −334.8 ppm as predominantly glutamate.

atom % excess and as total amount of ^{15}N incorporated, corrected for pool size (nmol/g fresh wt). In the control cells label was detected in all of the amino acids analyzed except for histidine and proline. The labeling data for glutamine and glutamate are particularly interesting. The high ^{15}N incorporation into glutamine (nmol/g fresh wt) relative to the low atom % excess is explained by the fact that glutamine accounts for 12% of the total amino acid pool, whereas glutamate is only 4%. The glutamine label was found predominantly in the amide nitrogen. Although label was present in the amino-N this represented less than 1 atom % excess. Levels below 1 atom % excess cannot be accurately determined by this method. This distribution of label suggests that there is a small, metabolically active pool of glutamine in the cytosol and a large, inactive pool in the vacuole. The glutamate labeling suggests that compared to the glutamine there is a large, metabolically active pool of this amino acid. The fact that glutamate showed such a high level of labeling throughout the 24 h suggests that some assimilation might be occurring via GDH, but the results for the MSO treated cells show that there was no incorporation of [^{15}N]ammonium into gluta-

Table II. *Incorporation of ^{15}N Label into Amino Acids after 5 h Treatment with [^{15}N]Ammonium in Presence and Absence of MSO*

Cells were cultured in the presence of 2 mM [^{15}N]ammonium chloride ± 1 mM MSO.

Amino Acid	Incorporation of ^{15}N		
		Control	1 mM MSO
	atom % excess	*nmol/g fresh wt*	*atom % excess*
Glutamate	24	190	0
Glutamine—amino	<1	<23	0
—amide	6	140	0
GABA	16	240	0
Alanine	24	150	0
Arginine	5	120	0
Serine	12	80	0
Methionine	13	80	0
Glycine	10	50	0
Valine	6	50	0
Aspartate	12	50	0
Leucine	13	50	0
Isoleucine	9	50	0
Threonine	4	40	0
Phenylalanine	11	40	0
Asparagine—amino	3	30	0
—amide	0	0	0
Tyrosine	3	12	0
Proline	0	0	0
Histidine	0	0	0

mate, when GS was inhibited. After 24 h the total label in the control cells, as determined by ANCA-MS, was 8.1 μmol ^{15}N/g fresh wt, of which only 0.2 μmol/g fresh wt was present as soluble amino acids.

In addition to preventing the incorporation of ^{15}N into amino acids, MSO also affected the concentration of ammonium in the medium (Table III). In control cells the ammonium concentration dropped from 1.6 mM to 0 over 12 h, representing an uptake rate of 1.3 μmol h^{-1}g fresh wt^{-1}. Measurements of cell ammonium also showed that there was very little residual ammonium in the cells, suggesting that ammonium was assimilated as it was taken up. In the MSO treatment ammonium was released into the medium at a rate of 0.8 μmol h^{-1}g fresh wt^{-1} during the period from 6 to 24 h. Analysis of the total cell protein (Table III) suggests that this ammonium was derived from protein turnover. The control cells showed a net gain to protein of 1.38 μmol N h^{-1}g fresh wt^{-1} corresponding to an uptake of 1.3 μmol N h^{-1}g fresh wt^{-1} as ammonium from the medium. In contrast over the same period the MSO cells showed a net loss from protein of 1.75 μmol N h^{-1}g fresh wt^{-1} and this was recovered as 1.14 μmol N h^{-1}g fresh wt^{-1} ammonium in the medium and cells.

These results show that despite the high levels of GDH present in carrot cells ammonium assimilation occurs solely via the GOGAT cycle. Moreover, GS activity is high during the period of rapid nitrogen assimilation and declines during the stationary phase, GDH on the other hand is lowest during the period of nitrogen assimilation and highest when sucrose is limited. The effect of carbon supply on GDH activity has been much studied and there is agreement that sugars exert a regulatory effect on this enzyme. In general GDH levels rise in response to carbon limitation and this effect is reversed by the addition of various sugars (16, 21). The link between GDH activity and carbon starvation is therefore well established.

It has been suggested (18) that MSO might affect the metabolism and transport of 2-oxoglutarate and thereby exert a substrate limitation effect on GDH. To investigate this possibility, the effect of adding 10 mM 2-oxoglutarate to both the control and MSO treatments was determined. Table III shows that the addition of 2-oxoglutarate had no effect on the production of ammonium by these cells, suggesting that GDH is not prevented from assimilating ammonium by insufficient 2-oxoglutarate.

The catabolism of protein and the production of ammonium suggested the possibility that GDH was active in the catabolism of glutamate. This was investigated by studying the metabolism of [^{15}N]glutamate using *in vivo* NMR, ANCA-MS, and conventional GC-MS.

Glutamate Metabolism

The metabolism of ^{15}N-labeled glutamate by cells pretreated with 2 mM MSO and AOA was followed over 24 h using *in vivo* ^{15}N NMR spectroscopy (Fig. 2). The increase in peak height and reduction in line width of the glutamate resonance between 2 and 9 h occur as glutamate is taken up from the

Table III. *Changes in Enzyme Activity, Ammonium Concentration in the Medium, and Total Cell Protein during 24 h Treatment with Ammonium in Presence and Absence of MSO and 2-Oxoglutarate*

Twenty-four hour treatments: ammonium (A), ammonium + MSO (AM), ammonium + 2-oxoglutarate (AO), ammonium + MSO + 2-oxoglutarate (AMO). Concentrations: 2 mM [^{15}N]ammonium chloride, 1 mM MSO, 10 mM 2-oxoglutarate. Values are the mean of three replicates ± SD.

Time	Treatment	Enzyme activity		Medium Ammonium Concentration	Total Cell Protein
		GS	GDH		
h		*nkat/mg protein*		*mM*	*mg/g fresh wt*
0		0.5 ± 0.015	18.0 ± 0.3	1.6 ± 0.01	15.5 ± 0.2
24	A	0.5 ± 0.015	9.8 ± 0.17	0	18.4 ± 0.7
	AM	0	10.8 ± 0	3.5 ± 0.04	11.8 ± 0.4
	AO	0.52 ± 0.012	9.0 ± 0	0	not determined
	AMO	0	10.6 ± 0.17	3.6 ± 0.04	not determined

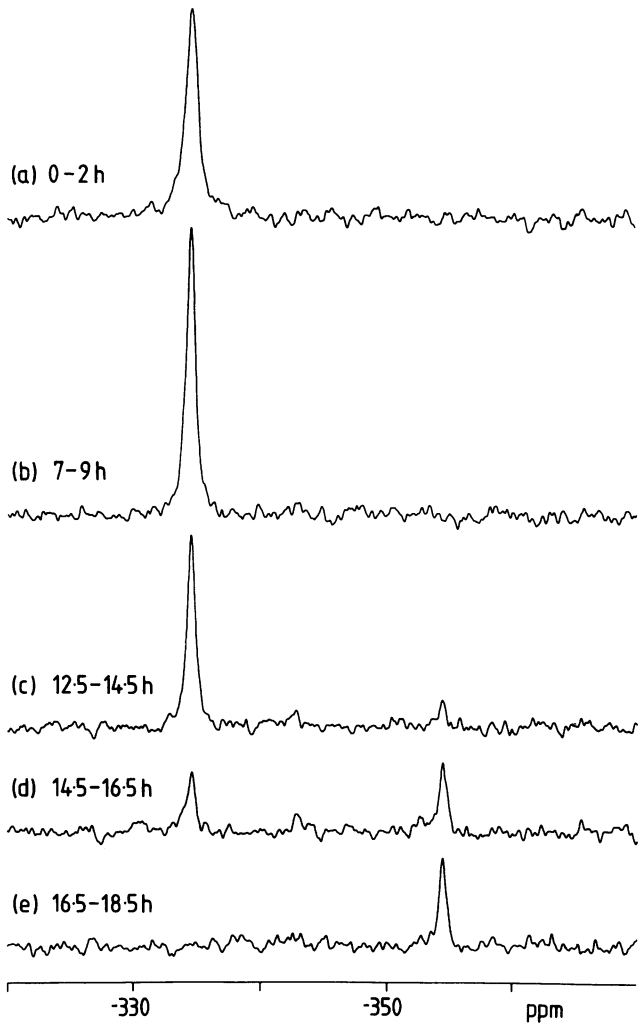

Figure 2. ^{1}H-coupled ^{15}N NMR spectra of 11 d carrot cells following the addition of 20 mM [^{15}N]glutamate. The spectra were obtained in two hours over the time intervals indicated. The increase in peak height and reduction in line width of the glutamate resonance (−334.8 ppm) between (a) and (b) was due to the uptake of glutamate from the external medium at pH 5.6 to the cytoplasm at pH 7.6. The properties of the ammonium resonance (−354.6 ppm) are discussed in the text.

medium into the cells. Ammonium was detected at 13.5 h and its concentration increased over the next 4 h, during this period there was a corresponding decrease in the glutamate resonance. The intensity of the final ammonium signal represented 15 to 30% of the original glutamate signal. However, the exact measurement of the fraction of the label that accumulated in ammonium is complicated by the fact that the observed signal probably only represents the cytoplasmic ammonium fraction as a result of the pH dependence of the ammonium linewidth (R Lee, RG Ratcliffe, submitted for publication). This experiment has been reproduced with two different cell culture lines. ^{1}H-decoupled spectra showed that GABA also became labeled (data not shown). The absence of the remaining amino acids from these spectra is due to their concentrations being below the detection limits of *in vivo* NMR.

A similar experiment involving treatment with 5 mM [^{15}N] glutamate in the presence or absence of either 1 mM MSO or 1 mM AOA was analyzed using conventional GC-MS and ANCA-MS. GS was completely inhibited after 6 h in the MSO treatment and remained high in the control and AOA treatments, and GDH activity decreased by 40% over 12 h in all three treatments (Table IV). The uptake of [^{15}N]glutamate was reduced by 25% by MSO treatment but was not affected by AOA (Table V). In the MSO treatment 10% of the ^{15}N label was recovered as [^{15}N]ammonium in the medium. This corresponds to 12% of the total ammonium produced by the cells. No ammonium was detected in the medium in either the control or the AOA treatment. The ^{15}N label in ammonium only includes that secreted into the medium and not the ammonium present in the cells. It was not possible to measure the labeling of the cell ammonium because the concentration was below the detection limit of the ANCA-MS. Incorporation of label into soluble amino acids was reduced by 50% in the MSO treatment compared to the control (Table VI). As a proportion of label taken up, soluble amino acids accounted for: control 29%, AOA treatment 23%, MSO treatment 19%. The proportion of label detected as insoluble nitrogen represented 63% to 65% of the total uptake for the three treatments.

In the AOA treatment ^{15}N labeling was significantly reduced in glutamate, methionine, aspartate, serine, and isoleucine and increased in GABA, histidine, and tyrosine (Table VI). The proportion of total glutamine label increased slightly in the AOA treatment, from 19% to 23%. Analysis of the position of the label showed that in the control the majority of label was present in the amino-N but treatment with AOA produced a more equal distribution, 55% amino-N, 45% amide-N. The label in the amide group suggests that [^{15}N] ammonium has been produced from the [^{15}N]glutamate and reassimilated via GS. In the MSO treatment, glutamate represents a larger percentage of the amino acid pool compared to the control and AOA treatment and there was no incorporation of ^{15}N into glutamine. MSO decreased the proportion of label in GABA and alanine and increased the proportion in the remaining amino acids, especially leucine, isoleucine, phenylalanine, aspartate, and arginine.

Table IV. *Changes in Enzyme Activity and Medium Ammonium Concentration during 12 h Treatment with [^{15}N]Glutamate in Presence of MSO, AOA, and without Inhibitors*

Cells were cultured in the presence of 5 mM [^{15}N]glutamate + 1 mM MSO or 1 mM AOA. Values are the mean of three replicates ± SD.

Time	Treatment	Enzyme Activity		Medium Ammonium
		GS	GDH	
h		*nkat/mg protein*		*mM*
0		1.2 ± 0.03	16.8 ± 0.3	0.25 ± 0.01
12	Control	1.0 ± 0.03	10.2 ± 0.2	0.2 ± 0.01
	AOA	1.2 ± 0.03	10.3 ± 0.2	0.19 ± 0.01
	MSO	0	10.7 ± 0.2	1.57 ± 0.07

Table V. *Distribution of ^{15}N in Cells after 12 h Incubation with [^{15}N]Glutamate*

Cells were cultured in the presence of 5 mM [^{15}N]glutamate ± 1 mM MSO or 1 mM AOA.

Sample	^{15}N Incorporation					
	Glutamate uptake	Ammonium in medium	Soluble amino acids	Insoluble nitrogen	Total recovered	Percent recovered
	μmol ^{15}N/g fresh wt					
Control	19.8	0	5.8	12.7	18.5	93
AOA	21.0	0	4.8	13.2	18.0	86
MSO	15.0	1.5	2.9	9.9	14.3	95

These experiments offer strong evidence to support the hypothesis that GDH is involved in the catabolism of glutamate. The production of [^{15}N]ammonium from [^{15}N]glutamate has been demonstrated both *in vivo* by NMR and *in vitro* using the ANCA system. In the presence of GS inhibitors ammonium can be detected in the media, whereas if GS is active, ammonium produced by this reaction is reassimilated into the amide of glutamine. The labeled ammonium recovered from the medium accounted for 10% of the label taken up by the cells.

CONCLUSIONS

These results demonstrate that GDH is active in the catabolism of glutamate in higher plants. With NMR it has been possible to demonstrate both ammonium assimilation and glutamate catabolism *in vivo*. This technique has shown that ammonium is first incorporated into the amide of glutamine offering further support for the GOGAT cycle as the major route of ammonium assimilation in higher plants. These studies also provide evidence that GDH is not involved in ammonium assimilation. There is no assimilation of ammonium under conditions where GS is inhibited even when the cells are supplied with excess 2-oxoglutarate. The glutamate metabolism experiment shows clearly that glutamate is catabolized to ammonium and 2-oxoglutarate. If GS is active this ammonium is detected in the amide-N of glutamine and if GS is inhibited ammonium is released into the medium.

GDH appears to catalyze the oxidation of glutamate in response to a deficiency of carbon. Under conditions of carbon limitation GDH activity in carrot cells increases by a factor of ten. The conclusion from these experiments is that the primary role of GDH is the oxidation of glutamate, thus ensuring sufficient carbon skeletons for effective functioning of the TCA cycle under conditions where carbon is limited. This catabolic role for GDH implies an important regulatory function in carbon and nitrogen metabolism.

Table VI. *Incorporation of ^{15}N Label into Amino Acids after 12 h Treatment with [^{15}N]Glutamate in Presence and Absence of Inhibitors*

Cells were cultured in the presence of 5 mM [^{15}N]Glutamate ± 1 mM MSO or 1 mM AOA. Percentages represent the proportion of the total ^{15}N labeled pool present as each amino acid.

Amino Acid	Control		^{15}N Incorporation			
			AOA		MSO	
	μmol/g fresh wt	%	*μmol/g fresh wt*	%	*μmol/g fresh wt*	%
Glutamate	0.57	9.8	0.17	3.6	0.48	16.6
Glutamine—amino	0.99	17.1	0.61	12.8	0	0
—amide	0.11	1.9	0.5	10.5	0	0
Alanine	0.67	11.6	0.57	11.9	0.19	6.6
GABA	1.93	33.3	2.05	42.9	0.71	24.5
Valine	0.14	2.4	0.13	2.7	0.1	3.4
Leucine	0.1	1.7	0.08	1.6	0.09	3.1
Isoleucine	0.12	2.1	0.06	1.3	0.13	4.5
Methionine	0.1	1.7	0.04	0.8	0.03	1.0
Serine	0.32	5.5	0.15	3.1	0.2	6.9
Threonine	0.26	4.5	0.18	3.8	0.18	6.2
Phenylalanine	0.04	0.7	0.04	0.8	0.04	1.4
Aspartate	0.41	7.1	0.07	1.5	0.45	15.5
Asparagine—amino	0.02	0.5	0.04	0.8	0.04	1.4
—amide	0	0	0	0	0	0
Tyrosine	0.01	0.1	0.03	0.6	0.01	0.3
Histidine	0	0	0.06	1.3	0.03	1.0
Arginine	0	0	0	0	0.22	7.6
Proline	0	0	0	0	0.22	0
Total	5.79	100	4.78	100	2.9	100

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